Title: Inorganic Particulate Matter Modulates Non-Typeable Haemophilus Influenzae Growth: A Link Between Chronic Bacterial Infection and Geogenic Particles.

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Authors contributions: Mr Williams acquired all data and prepared the draft manuscript. Dr Tristram provided extensive insight and knowledge into bacterial behaviour, data analysis and

draft editing. Professor Zosky conceived the project, assisted with experimental design and data analysis and edited the manuscript.

Abstract

Australian Aboriginal populations have unacceptably high rates of bronchiectasis. This disease burden is associated with high rates of detection of pathogenic bacteria; particularly Non-typeable *Haemophilus influenzae* (NTHi). While there is evidence to suggest that exposure to inorganic particulate matter (PM) is associated with worse respiratory infections, no studies have considered the direct effect of this PM on bacterial growth. Nine clinical isolates of pathogenic NTHi were used for this study. Isolates were exposed to two common iron oxides, haematite (Fe_2O_3) or magnetite (Fe_3O_4), or quartz (SiO_2), as the main constituents of environmental inorganic PM. NTHi isolates were exposed to PM with varying levels of heme to identify whether the response to PM was altered by iron availability. The maximal rate of growth and maximum supported growth were assessed. We observed that inorganic PM was able to modify the maximal growth of selected NTHi isolates. Magnetite and quartz were able to increase maximal growth while haematite could both increase and suppress the maximal growth. However, these effects varied depending on iron availability and on the bacterial isolate. Our data suggests that inorganic PM may directly alter the growth of pathogenic NTHi. This observation may partly explain the link between exposure to high levels of crustal PM and chronic bacterial infection in Australian Aboriginals.

Keywords: Lung infection, Aboriginal children, Bronchiectasis, Heme, Iron

Introduction

Bronchiectasis is a chronic lung disease characterised by an irreversible dilation of the small airways that causes significant morbidity and reduced life-expectancy (Loebinger et al. 2009). While, in many cases, the pathogenesis of bronchiectasis is unclear, there is evidence to suggest a link between exposure to air pollution and the risk and severity of disease. For example, exposure to traffic-derived air pollution in urban centres is linked to increased mortality in patients with bronchiectasis (Goeminne et al., 2014) and the risk of acute exacerbations of bronchiectasis (Goeminne et al., 2018) suggesting that environmental factors are important in disease pathogenesis. Interestingly, Australian Aboriginal people, who often live in communities in remote and rural settings outside of urban centres where levels of traffic-related pollution are low, experience a disproportionate burden of bronchiectasis, almost three times higher than the non-Aboriginal population (Blackall et al. 2018). Furthermore, Aboriginal children suffer from one of the highest prevalence's of non-cystic fibrosis bronchiectasis in the world (Chang et al. 2002). While there are a range of factors associated with social disadvantage that may be contributing to this unacceptably high burden of disease (Australian Bureau of Statistics 2017; Australian Institute of Health and Welfare 2016; Melody et al. 2016; O'Grady and Chang 2010; O'Grady et al. 2010), emerging evidence suggests environmental factors also contribute (Clifford et al. 2015). For example, geogenic (earth-derived) particulates are abundant in remote Aboriginal communities (Clifford et al. 2015; Melody et al. 2016) and are correlated with the severity of lower respiratory tract infections in these communities (Shepherd et al., 2019).

Bronchiectasis typically develops as a result of uncontrolled, chronic bacterial infection in the airways (King 2009). Almost 90% of Aboriginal people with bronchiectasis return a positive result for the presence of pathogenic bacteria in sputum samples, predominately Non-typeable

97 *Haemophilus influenzae* (NTHi) (Blackall et al. 2018; Pinto et al. 2016; Pizzutto et al. 2017;
98 Watson et al. 2006). There is a growing body of evidence that inorganic particulates can
99 contribute to the risk and severity of bacterial infections in the lung. For example,
100 epidemiological studies demonstrate that occupational exposure to mineral dusts increases the
101 risk of bacterial pneumonia (Koh et al. 2011; Toren et al. 2011). In line with this, populations
102 exposed to wind-blown dust from iron ore stockpiles have greater rates of hospitalisation for
103 respiratory conditions (Government of Western Australia 2016; Mullan et al. 2006),
104 particularly respiratory infections (Government of South Australia 2007). Inhalable particulate
105 matter (PM) from a range of sources has been shown to modify the response to NTHi *in vitro*
106 and *in vivo* (Clifford et al. 2016; Zarcone et al. 2017; Ghio 2014). However, these studies have
107 primarily focussed on the host response to NTHi and have not considered the direct effect of
108 PM on bacterial growth.
109
110 There is some evidence to suggest that iron nanoparticles can directly influence bacterial
111 growth, however, the response varies considerably depending on the type of bacteria and
112 physico-chemical characteristics of the particles used (Kim et al. 2017; Liu and Vipulanandan
113 2013). To date, no studies have characterised the response of NTHi to particulates in the coarse
114 fraction respirable PM (1-10μm in diameter). As a result, the contribution of the direct effects
115 of inorganic PM inhalation to NTHi infection are yet to be determined. The geogenic PM that
116 Aboriginal children are exposed to is dominated by high levels of silica (quartz; SiO_2) and iron
117 oxide (haematite; Fe_2O_3 and magnetite; Fe_3O_4) (Zosky et al. 2014a; Zosky et al. 2014b).
118 Therefore, the aim of this study was to assess the effect of these particles on NTHi growth.
119
120
121

Methods

Particle preparation

Commercially available standard preparations of dry magnetite (Fe_3O_4; Sigma-Aldrich 310069), haematite (Fe_2O_3; Sigma-Aldrich 310050) and α-quartz (SiO_2; NIST 1878B) were used for bacterial growth experiments. NTHi responses to these particles were compared to particle-free bacterial growth. Particle samples were exposed to ultraviolet (UV) light for two hours prior to experimentation to remove any existing bacterial or endotoxin contamination.

Bacterial isolates

We used 4 clinical NTHi isolates from the lower respiratory tract, 3 from ear swabs or effusions and 2 from upper respiratory swabs of the oropharynx. The isolates were identified by the original laboratories where the isolates were collected as *H. influenzae*, without knowledge of this study, based on colonial morphology and X + V factor dependence, and assigned clinical significance based on criteria that involved relevant clinical history, dominant growth on primary culture and supporting microscopy of the clinical specimens. All isolates were retrospectively identified in this study as *H. influenzae* using a polymerase chain reaction (PCR) algorithm for key species marker genes including *fucK*, *hpd*, and sodC as previously described (Witherden and Tristram 2013). Isolates were chosen to span secondary characteristics including invasion rate of bronchial epithelial cells (Table 1).

Conditions, exposures & growth curves

To quantitatively analyse bacterial growth, growth curves of each isolate were compared in two ways. Firstly, we assessed differences in maximum rate of growth. These data were generated from the highest rate of exponential growth observed for each isolate under each heme condition and particle exposure. Secondly, we assessed for the maximum supported

147 population, represented as the highest density of bacteria at any timepoint across the assessment
148 period.
149
150 NTHi has a dependence on heme availability (Stojiljkovic and Perkins-Balding 2002).
151 However, the strength of this dependency may vary between isolates. Initially, the effect of
152 heme status on rate of growth was compared between replete (15 µg/mL heme), limited (2
153 µg/mL heme) and deplete (0 µg/mL heme) heme conditions in the absence of particles. During
154 routine culture, NTHi was grown under optimal conditions on Chocolate agar or in Brain Heart
155 Infusion (BHI; Oxoid, UK) broth supplemented with Vitox and Haemophilus Test Media
156 (HTM; Oxoid, UK). Under each heme condition, isolates were exposed to 0 or 50 µg/mL of
157 particles (SiO_2, Fe_2O_3 or Fe_3O_4). Exit cultures were utilised to confirm the absence of bacterial
158 contamination.
159
160 Bacteria were grown at 37ºC on an orbital shaker (220 rpm) in atmospheric CO_2. Absorbance
161 (optical density; OD) of the cultures was measured at 600 nm on the BioPhotomer spectrometer
162 (Eppendorf, Germany), hourly for 14 hours (Fig 1). This was sufficient to observe a complete
163 growth curve for each isolate. To account for the colour pigment of the particles, standard
164 curves were formulated for each isolate under each particulate exposure condition. The optical
165 density measured by absorbance was then transformed into colony forming units (CFU)/mL by
166 removing the colorimetric effects of the particles by comparison of OD_{600} to CFU/mL standard
167 curves determined for each particulate and bacterial isolate.
168
169 *Statistical analysis*
170 Comparisons between groups were made using two-way repeated measures ANOVA. When
171 significance was determined for the main factors by ANOVA, a Holm-Sidak post-hoc test was

used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD) and values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

Results

The effect of heme status

The main effects of heme status on the rate of growth and maximum growth in all isolates are summarised in Table 2.

Rate of growth

When compared to replete heme conditions, isolates Ci8 ($p = 0.010$), Ci43 ($p < 0.001$) and L227 ($p < 0.001$) grew slower under deplete heme conditions. Interestingly, heme availability did not alter rate of growth in isolates NF3 ($p = 0.067$), Ci16 ($p = 0.148$), Ci34 ($p = 0.282$), Ci37 ($p = 0.161$), L267 ($p = 0.271$) or L341 ($p = 0.236$).

Of the heme-dependent strains, isolate Ci43 exhibited an intermediate level of growth under limited heme conditions when compared to growth under replete ($p < 0.001$) and deplete heme conditions ($p = 0.011$). In contrast, while there was a difference between replete and deplete conditions in the Ci8 and L227 isolates, the rate of growth was not modified by limited heme (Ci8, $p = 0.239$; L227, $p = 0.142$).

Maximum growth

When compared to replete heme conditions, heme depletion reduced maximal growth in the Ci8 ($p = 0.010$), Ci16 (Fig 2A; $p = 0.007$), Ci43 ($p < 0.001$), L227 ($p < 0.001$) and L267 (Fig 2B; $p = 0.007$) isolates. We observed no difference between replete and heme deplete conditions in maximal growth for the Ci34 ($p = 0.121$), Ci37 ($p = 0.053$), NF3 ($p = 0.067$) and L341 ($p = 0.297$) isolates. Limited heme resulted in significantly reduced growth compared to replete conditions for the Ci37 ($p = 0.046$), Ci43 ($p < 0.001$), L227 ($p < 0.001$) and L267 (Fig

2B; p = 0.008) isolated, but not for Ci8 (p = 0.138), Ci16 (Fig 2A; p = 0.150), Ci34 (p = 0.430), NF3 (p = 0.069) or L341 (p = 0.297).

The effects of particle exposure

The main effects of particle exposure on the rate of growth and maximum growth in all isolates are summarised in Table 3.

Rate of Growth

Rate of growth was not altered by particle exposure for the NF3 (p = 0.227), Ci8 (p = 0.400), Ci16 (p = 0.420), Ci43 (p = 0.511), L227 (p = 0.132), L267 (p = 0.336) or L341 (p = 0.163) isolates. Interestingly, iron oxide altered the rate of growth for isolates Ci34 and Ci37. Magnetite exposure increased rate of growth of Ci34 (p = 0.011). Under replete heme, the Ci37 isolate demonstrated significantly slower growth when exposed to magnetite (p = 0.004) or haematite (p = 0.003). In contrast, under deplete conditions magnetite increased rate of growth for the same isolate (p < 0.001). Quartz had no effect on rate of growth of any isolate (p > 0.05 for all comparisons).

Maximum Growth

There was no effect of particulates on maximum growth of the NF3 (p = 0.227), Ci8 (p = 0.471), Ci16 (p = 0.064), L227 (p = 0.052) or L267 (p = 0.164) isolates. Quartz increased the maximal growth of L341, regardless of heme status (p < 0.001). Magnetite increased growth of isolate Ci34 under both the limited (Fig 3; p < 0.001) and deplete heme conditions (Fig 3; p = 0.001) and the Ci37 isolate with deplete heme (p = 0.046). Regardless of heme, magnetite increased the maximum growth of Ci43 (p < 0.001). In comparison, haematite increased

maximal growth of the Ci34 isolate under limited (Fig 3; $p < 0.001$) and deplete heme (Fig 3; $p < 0.01$) but suppressed the growth of L341 ($p = 0.014$).

Discussion

Remote Australian Aboriginal communities are exposed to high levels of inorganic particulate matter (Shepherd et al. 2019), dominated by silica and iron oxides (Zosky et al. 2014b), and have a disproportionate burden of chronic respiratory infections (O'Grady & Chang 2010). The present study aimed to identify whether silica or iron oxide PM can directly modify the growth of NTHi as one of the dominant forms of pathogenic respiratory bacteria in these communities. We observed that inorganic PM was able to modify the maximal growth of selected NTHi isolates with the effect varying depending on iron availability. Interestingly, the effects were dependent on the bacterial isolate.

Our study highlights the heterogeneous nature of the bacterial response, even within a single species. Initial testing sought to identify the sensitivity of the isolate to heme. Two isolates were unaffected by heme availability, demonstrating uninhibited growth in the absence of heme. Given the complete dependence of NTHi on heme, this observation is most likely due to the ability of the organism to store heme during pre-exposure growth. While most of the isolates demonstrated diminished growth in response to lowering heme concentrations as expected (Whitby et al. 2009; Whitby et al. 2013), it clearly demonstrates the adaptive ability of human pathogens to operate under extremely low iron conditions (Cassat and Skaar 2013; Hood and Skaar 2012; Weinberg 1975).

The effects of PM on NTHi growth were heterogenous. While the mechanism is unclear, it is possible that bacterial sources of iron can be altered by environmental factors. Importantly, magnetite retains surface reactivity and protein binding affinity (Kang et al. 2007; Sun et al. 1998). In this context, magnetite reduction is optimal at pH 5-6 and 22-37°C, which is consistent with our bacterial culture parameters and most biological enzymatic processes

256 (Kostka and Nealson 1995). Magnetite ($Fe^{2+}Fe_2^{3+}O_4^-$) can undergo reduction to form Fe^{2+}, H_2O
257 and HCO_3^- molecules (Kostka and Nealson 1995). Interestingly, this reduction process is
258 suppressed in the presence of oxygen meaning this particulate would be relatively inert in the
259 lung. We propose that partial reduction of magnetite under the conditions we used led to a
260 moderate increase in Fe^{2+} which promoted bacterial growth. NTHi can utilise a range of heme
261 containing proteins (Choby and Skaar 2016). However, none of these include free Fe^{2+}, with
262 even relatively low levels of free iron being highly toxic. Thus, it is possible that the small
263 increase in available Fe^{2+} from partial magnetite dissociation resulted in advantageous bacterial
264 growth conditions by saturation of iron-containing proteins such as transferrin or ferritin.
265
266 In comparison, quartz is coated in oxygen-containing surface functional groups. Given their
267 chemical nature, these silanol functional groups retain a high affinity for Fe^{3+} (Ghio et al. 1992;
268 Ghio et al. 1994; Ghio et al. 2016). Despite varying chemically, haematite also possesses the
269 ability for Fe^{2+} sorption (Jeon et al. 2001; Larese-Casanova and Scherer 2007). It is unclear
270 what effect this may have on the bioavailable iron. However, this may explain why we observe
271 less consistent, and sometimes reduced growth, in the presence of these particulates, in contrast
272 to the effect of magnetite. Thus, the effects of haematite are somewhat unexpected as the
273 particle can undergo reduction and is often used for iron fertilization in water ecosystems to
274 boost microbial growth (Fan et al. 2006). However, this may reflect the specific condition of
275 the *in vitro* environment we used.
276
277 We must acknowledge that the present study has some limitations. In particular, we have not
278 established a mechanism by which the PM altered growth. Secondly, while we have attempted
279 to cover a range of different isolates, we were not able to attribute variation in response to any
280 specific characteristic of bacterial behaviour. Despite these limitations the present study

281 demonstrates that pathogenic strains of NTHi can be directly influenced by inorganic PM.
282 Future research should focus on linking the responses we observed with the host cell response
283 and probe the possible mechanisms linked to the variable growth as a result of PM exposure.
284 NTHi infection leads to several diseases of the ear, upper and lower airways (Foxwell et al.
285 1998) and is associated with irreversible chronic respiratory diseases such as bronchiectasis
286 (Blackall et al. 2018) in vulnerable populations. Our data are the first to provide evidence that
287 inorganic, environmentally relevant, PM matter can aid bacterial growth. Our work implies
288 that reducing exposure to PM from geogenic sources may improve respiratory health in at risk
289 communities.
290

Abbreviations:

CFU/mL; Colony forming units/ millilitre

NTHi; Non-typeable Haemophilus influenzae

PM; Particulate matter

SD; Standard deviation

Declarations:

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no competing interests to declare.

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Authors contributions

Mr Williams acquired all data and prepared the draft manuscript. Dr Tristram provided extensive insight and knowledge into bacterial behaviour, data analysis and draft editing. Professor Zosky conceived the project, assisted with experimental design and data analysis and edited the manuscript.

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Figure Legends

Figure 1: Example growth curve of isolate Ci34 under limited heme and in response to particulates. Growth is displayed as colony forming units/mL (CFU/mL) over 14 hours of measured growth. Magnetite and haematite both increased maximal growth compared to control ($p < 0.001$). Data are presented as mean(SD) from 6 independent experiments with * indicating $p < 0.05$.

Figure 2: Maximum growth of isolates Ci16 (A) and L267 (B) in response to varying levels of heme. Deplete heme resulted in significantly decreased maximum growth (CFU/mL) when compared to replete heme for isolate Ci16 (A), but not isolate L267 (B). Data are shown as absolute CFU/ml values. Data are presented as mean(SD) from 6 independent experiments with * indicating $p < 0.05$.

Figure 3: Maximum growth of isolate Ci34 in response to replete, limited and deplete heme availability as well as particulate exposure. Magnetite significantly increased growth compared to control under limited ($p < 0.001$) and deplete ($p = 0.001$) heme. Haematite increased growth under limited ($p < 0.001$) and deplete ($p = 0.004$) heme. Data are presented as mean(SD) from 6 independent experiments with * indicating $p < 0.05$.

Tables

Table 1: H. influenzae characteristics, including site of isolation and invasion rate of bronchial epithelial cells.

Identifier	Site of Isolation	% Invasion of bronchial epithelial cells Mean (SD)
Ci8	Sputum	Moderate- 14.79 (9.11)
Ci16	Sputum	Low- 0.47 (0.15)
Ci34	Sputum	Low- 0.01 (0.01)
Ci43	Sputum	Low- 1.84 (0.17)
Ci37	Ear	Low- 0.01 (0.00)
L267	Ear	High- 36.69 (17.61)
L341	Ear	Moderate- 15.25 (6.25)
NF3	Oropharynx	Low- 2.59 (1.28)
L227	Oropharynx	High- 33.90 (6.31)

Table 2: The response of NTHi isolates to limited and deplete heme availability when compared to the response of NTHi in replete heme conditions.

Bacterial Characteristics			**Rate of Growth**		**Maximum Growth**	
Isolate	**Origin**	**Invasiveness**	**Limited**	**Deplete**	**Limited**	**Deplete**
NF3	Oropharynx	Low	-	-	-	-
Ci8	Sputum	Moderate	-	Decrease	-	Decrease
Ci16	Sputum	Low	-	-	-	Decrease
Ci34	Sputum	Low	-	-	-	-
Ci37	Ear	Low	-	-	Decrease	-
Ci43	Sputum	Low	Decrease	Decrease	Decrease	Decrease
L227	Oropharynx	High	-	Decrease	Decrease	Decrease
L267	Ear	High	-	-	Decrease	Decrease
L341	Ear	Moderate	-	-	-	-

Table 3: The response of NTHi isolates to quartz, haematite of magnetite particulates. The responses listed are within replete heme availability unless otherwise specified.

Bacterial Characteristics			**Rate of Growth**			**Maximum Growth**		
Isolate	**Origin**	**Invasiveness**	**Quartz**	**Haematite**	**Magnetite**	**Quartz**	**Haematite**	**Magnetite**
NF3	Oropharynx	Low	-	-	-	-	-	-
Ci8	Sputum	Moderate	-	-	-	-	-	-
Ci16	Sputum	Low	-	-	-	-	-	-
Ci34	Sputum	Low	-	-	Increase	-	Increase (limited & deplete heme)	Increase (limited & deplete heme)
Ci37	Ear	Low	-	Decrease	Decrease (replete heme) and increase (limited heme)	-	-	Increase (deplete heme)
Ci43	Sputum	Low	-	-	-			Increase
L227	Oropharynx	High	-	-	-	-	-	-
L267	Ear	High	-	-	-	-	-	-
L341	Ear	Moderate	-	-	-	Increase	Decrease	-